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Cryopreservation of tissue-engineered epithelial sheets in trehalose

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ABSTRACT

Tissue-engineered epidermal membranes are useful for clinical wound healing. To facilitate these products in the clinic, optimized storage methods need to be developed. We studied the efficiency of extracellular trehalose at various concentrations for cryopreserving human tissue-engineered epidermal membranes compared with that of dimethyl-sulfoxide (DMSO) used by most organ banks for cryopreserving skin grafts and artificial skin substitutes. Keratinocyte (KC) viability, proliferation and marker expression following cryopreservation in trehalose were examined with similar results to those using DMSO. Trehalose concentration (0.4M) was optimized according to the described cellular activities following cryopreservation. Artificial epidermal substitutes were then cryopreserved in trehalose at the optimized concentration. Cell viability, growth factor secretion and wound healing properties of cryopreserved artificial epidermal substitutes using nude mice were examined and compared with those of DMSO cryopreservation. Cryopreservation with trehalose enhanced human KC viability in suspension and artificial skin substitutes. In addition, trehalose cryopreservation provided fast recovery of EGF and TGF-β1 secretion by KCs after thawing. When transplanted into nude mice, trehalose-cryopreserved artificial skin repaired skin defects in a similar manner to that of a non-cryopreserved control. Moreover, trehalose-cryopreserved artificial skin resulted in engraftment and wound closure that was significantly enhanced compared with that of DMSO-cryopreserved epidermal membranes. The results indicate that the use of trehalose improves cryopreservation of tissue-engineered epithelial sheets.

1. Introduction

Cultured epidermal allografts are used to treat a wide variety of skin defects ranging from burns to leg ulcers [1,2]. Early therapies were performed by using cultured KCs membranes [3,4]. However, these cultured cell sheets are very difficult to handle. The development of tissue engineering techniques has allowed biodegradable scaffolds to be used for epidermal graft engineering. Various biodegradable sheets have been developed as substrates to support KCs growth and improve the mechanical properties of grafts [5,6]. Biodegradation of grafted materials has resulted in wound healing in animal models and human patients [7,8]. By using chitosan-gelatin membranes (CGMs) seeded with allogeneic KCs, we previously demonstrated that healing at skin graft donor sites is promoted and long term hypertrophic scarring was reduced in 24 patients [9].

However, engineering of epidermal grafts is time consuming with several weeks required for cell expansion to obtain a transplantable graft. This may limit the use of these grafts in vital situations such as following trauma and severe burning. Therefore, there is a necessity to maintain large stocks of engineered grafts to ensure an on-hand supply that can meet unpredictable needs. This would require an efficient cryopreservation technique that can store engineered grafts for several months or years and be ready for use immediately after thawing.

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Although numerous protocols have been developed for cryopreserving human skin [10,11], there is little information on cryopreservation of tissue-engineered epidermal grafts. It has been demonstrated that cryopreserved split-thickness skin is protected in DMSO while frozen with high viability after thawing [12]. However, a low survival rate was observed upon thawing of KCs monolayers that were cryopreserved in DMSO. In addition, the epithelial barrier function of cryopreserved cultured skin substitutes, including human KCs and fibroblasts attached to collagen-glycosaminoglycan substrates, was impaired upon thawing after controlled-rate cryopreservation in DMSO [13]. Our own preliminary studies using the same protocol to cryopreserve KC-CGMs epidermal grafts resulted in a low survival rate (31.2%) (unpublished data). Therefore, efficient cryopreservation protocols for KC-CGMs epidermal grafts need to be developed.

Previous studies have demonstrated that trehalose, a non-permeating cryoprotectant, improves cell viabilities during cryopreservation of cell suspensions [14,15] and whole organs [15]. Our previous study [17] also showed that trehalose significantly improves the survival of human embryonic stem cells that are known to be difficult to cryopreserve. Trehalose combined with DMSO enhances the viability of cryopreserved fetal skin *in vitro* and significantly improves engraftment and wound closure *in vivo*. We theorize that trehalose may also improve cryopreservation efficiency of engineered KC-CGMs epidermal grafts. Therefore, we investigated KCs viability following cryopreservation in DMSO and trehalose, and evaluate *in vivo* engraftment and wound healing efficiency of trehalose-cryopreserved artificial skin substitutes by transplantation into a nude mouse model of wound healing.

2. Materials and methods

2.1. Human KCs isolation and culture

KCs were isolated from young human foreskins following circumcision using 0.24 U/mL dispase (Roch, Switzerland) and 0.05% trypsin-0.53 mM EDTA (Gibco, USA). Primary KCs were suspended in keratinocyte-serum free medium (KSFM, Gibco, USA) and seeded onto tissue culture plates (Falcon, USA) at $\sim 3.5 \times 10^4$ cells/m^2 in 10 mL complete medium and incubated at 37 °C with 5% CO_2. Culture medium was exchanged every other day. Upon reaching 70–80% confluence, KCs were trypsinized and passaged at a 1:3 split ratio. Third passage cells were used for experimental procedures.

2.2. Preparation of CGMs

An aqueous solution of chitosan (Qindao Haihui Biology Engineering Co., Ltd., Qingdao, China) and gelatin (Sigma, USA) was prepared by mixing a weight ratio of 7:3 (chitosan: gelatin) at 50 °C for 60 min. An N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (Sigma, USA) solution was then added for cross-linking. The solution was poured into the wells of 6-well plates and desiccated at 37 °C for 24 h. CGMs were washed several times with deionized water and then sterilized by Co^{60} at 25 kGy [18].

2.3. Human KCs culture on CGMs

Third passage KCs at 70–80% confluence were trypsinized, resuspended in medium and counted using a hemocytometer. Cell suspensions were diluted to the desired concentration and seeded onto CGMs (1 cm^2) at 5×10^4 cells/cm^2. Cell cultures were maintained with medium changes every other day until reaching 90% confluence. KC-CGMs were then used for cryopreservation experiments.

2.4. Cryopreservation solutions

Four cryopreservation solutions were prepared with the following formulations: (1) 0.2 M trehalose (Sigma, USA), 10% (v/v) DMSO (Sigma, USA), 50% (v/v) fetal bovine serum (FBS, SAFC, Australia) (Group 0.2 M T + D); (2) 0.4 M trehalose, 10% (v/v) DMSO, 50% (v/v) FBS (Group 0.4 M T + D); (3) 0.6 M trehalose, 10% (v/v) DMSO, 50% (v/v) FBS (Group 0.6 M T + D); and (4) 10% (v/v) (DMSO), 50% (v/v) FBS (Group DMSO, as a negative control). Cryopreservation solutions were diluted in KSFM and filter sterilized using a 0.22 μm filter (Millipore, USA).

2.5. Cryopreservation

2.5.1. Cell suspensions

Cells were harvested at 70–80% confluence and transferred to 2 mL cryovials containing 1.5 mL of the various cryopreservation solutions and kept at 4 °C for 30 min followed by placing into liquid nitrogen (LN_2). As a control, cell suspensions in the DMSO group were step frozen at 4 °C for 30 min, −20 °C for 2 h, −80 °C overnight and then placed into LN_2.

2.5.2. KC-CGMs

KC-CGMs (1 cm × 1 cm) were transferred to 2 mL cryovials containing 1.5 mL of the 0.4 M T + D cryopreservation solutions and kept at 4 °C for 30 min followed by placing into LN_2. The control group used stepped cryopreservation in DMSO medium as described.

2.5.3. CGMs

CGMs (8 cm × 8 cm) were transferred to 5 mL cryovials containing 4.5 mL of the 0.4 M T + D cryopreservation solutions and kept at 4 °C for 30 min followed by placing into LN_2. The control group used stepped cryopreservation in DMSO medium as described.

2.6. Thawing

2.6.1. Cell suspensions

After 1 month cryopreservation, samples were removed from LN_2 and immediately placed into a 37 °C water bath until almost completely thawed. Cell suspensions were transferred to sterile centrifuge tubes (15 mL, Falcon, USA) and diluted in 9 mL KSFM. After 5 min centrifugation at 300 *g*, cell pellets were resuspended in 1 mL KSFM. Resuspended cells were plated at 1×10^4 cells/well in 24-well plates for further analysis.

2.6.2. KC-CGMs

After 1 month cryopreservation, samples were thawed as described. Membranes were transferred to 6-well plates and washed three times with KSFM. KC-CGMs were cultured in KSFM for further analysis.

2.6.3. CGMs

After 1 month cryopreservation, samples were thawed as described. Membranes were transferred to 10 cm culture dish and washed three times with KSFM. CGMs were kept moist in saline for further analysis.

2.7. Cell viability assay

Cryopreserved KCs viabilities were determined by staining viable cells with the green fluorescent dye calcein AM and necrotic cells with the red fluorescent dye ethidium homodimer-1 (Live/Dead Viability/Cytotoxicity Assay Kit, Invitrogen Detection Technologies, USA) following the manufacturer's instructions. Images were recorded by a fluorescent microscope (Nikon, Japan) ($n = 9$ for each group).

Viable KCs percentages were assessed by propidium iodide (PI, Sigma, USA) staining and flow cytometric analysis (Beckman Coulter, USA) ($n = 9$ for each group). PI intercalates into double-stranded DNA and is excluded by viable cells but not necrotic cells. Cell pellets were resuspended in a PI solution (1 mg/mL in PBS) and protected from light at 4 °C until analyzed.

2.8. Cell proliferation assay

Proliferative capacities of cryopreserved and non-cryopreserved KCs were determined by daily DNA content assays for 9 days after initial plating ($n = 9$ for each group) as described elsewhere [19]. Briefly, cells were harvested at the various time points, mechanically lysed and then treated with 0.5 mL proteinase K (0.5 mg/mL, Sigma, USA) at 56 °C overnight. DNA content in the lysate was quantified spectrofluorometrically using Hoechst 33258 dye (Sigma, USA) by correlating to a standard curve. The DNA standard curve was generated by serial dilutions of known DNA concentrations from lysed keratinocytes.

2.9. Cell cycle analysis

Cell cycle phases were determined by DNA staining with PI ($n = 9$ for each group). In brief, cells were harvested, washed with PBS, fixed with 70% ethanol and stored at 4 °C for at least 24 h. Cells were washed again with PBS and then incubated with PI (50 μg/mL) and RNase (20 μg/mL) at 37 °C for 30 min. Cells numbers at various cell cycle phases were measured by a flow cytometer and analyzed using Multicycle software (Beckman Coulter, USA).

2.10. Immunocytochemistry

For pan-cytokeratin (pCK) and Cytokeratin 19 (CK19) staining, KCs in a stationary phase from each group were fixed for 30 min in ice-cold 4% formaldehyde and then incubated with permeabilization solution (0.3% Triton X-100) for

10 min followed by washing with PBS twice. pCK marker was detected with a mouse monoclonal antibody (1:100; Abcam, UK), and K19 was detected with a rabbit polyclonal antibody (1:100; Abcam, UK). For pCK, a secondary Alexa-Fluor® 488-labeled goat anti-mouse IgG (1:1000; Invitrogen, USA) was used for detection. For CK19, an Alexa-Fluor® 555-labeled goat anti-rabbit IgG (1:1000; Invitrogen, USA) was used for detection. For β1-integrin staining, KCs were prepared as described without permeabilization and incubated with an mouse anti-β1-integrin antibody (1:100; Chemicon, USA). A secondary Alexa-Fluor® 488-labeled goat anti-mouse IgG (1:1000; Invitrogen, USA) was used for detection ($n = 9$ for each group).Cells were observed and imaged under a fluorescence microscope.

2.11. KCs viability and proliferation on CGMs

KCs viabilities on CGMs were assessed at days 0 and 3 by calcein AM and ethidium homodimer-1 staining as previously described ($n = 9$ for each group). Nine random areas on KC-CGMs were counted for each group to determine KCs viability at each time point.

2.12. Scanning electronic microscopy (SEM)

The CGMs surface with attached KCs and deposited extracellular matrix was visualized after thawing at days 1 and 3. KC-CGMs were fixed in 2.5% glutaraldehyde, post-fixed in 0.1% osmium tetroxide (OsO_4), dehydrated via an ethanol series with hexamethyldisilazane (HMDS) and air-dried at room temperature. Samples ($n = 9$ for each group) were sputter-coated with gold and examined by SEM (Philips XL-30, Netherlands). CGMs without cells after thawing were also analyzed by SEM.

2.13. Quantitative analysis of growth factor secretion from KC-CGMs

Growth factors (EGF and TGFβ-1) secreted from KC-CGMs were measured in triplicate from conditioned medium by enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions (R&D, USA) ($n = 9$ for each group). ELISAs were read on a microplate reader at 450 nm and quantified using standards provided in the kits.

2.14. In vivo mouse wound healing model

Eighteen male BALB/C nude mice were purchased from SLAC National Rodent Laboratory Animal Resources (Shanghai, China). Animal experimentation protocols were approved by the institutional review committee of the Shanghai Jiao Tong University School of Medicine. Mice were anesthetized with intraperitoneal injections of pentobarbital sodium (20 mg/kg body weight). Two circular full-thickness 1 cm diameter wounds were created on the backs of mice. Non-cryopreserved fresh KC-CGMs (culture Day 3), thawed KC-CGMs with DMSO and 0.4 M T + D and CGMs alone groups were placed onto the wound area and held by 5–0 nylon sutures at the corners ($n = 9$ for each group). Grafts were covered with Vaseline (Shaoxing Zhende surgical dressing Co., Ltd., Shaoxing, China) gauze and adhesive bandages. Animal behavior and bandage integrity were monitored throughout the experiment. Wounds were evaluated at days 0, 4, 7, 11 and 14 post-operation. Images were recorded with a digital camera (Canon, Japan) to visualize the wound. The wound area was measured by tracing the wound margin and calculated using Image-Pro Plus Software (version 5.0; Media Cybernetics LP, Silver Spring, MD). Investigators measuring samples were blinded to groups and treatment. Wound closure percentage was calculated as: (original wound area − actual wound area)/original wound area × 100%. Animals were euthanized at day 14 and skin grafts embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) followed by snap freezing for immunohistology and H&E staining. Human KCs engraftment was analyzed by immunohistochemical staining of cryosections for HLA-ABC antigens. HLA-ABC was detected with a mouse monoclonal antibody (1:100; Chemicon, USA), and a secondary Alexa-Fluor® 488-labeled goat anti-mouse IgG (1:1000; Invitrogen, USA) was used for detection.

2.15. Biomechanical analysis

For biomechanical analysis, CGMs from all groups were cut into strips (1 cm × 8 cm) and kept moist in saline. CGMs strips were analyzed using a biomechanical analyzing instrument (Instron, Canton, M.A.) to measure elongation at breakage and tensile strength. Briefly, CGMs were fixed in pneumatic grips at both ends and a stretching force applied at 5 mm/min until CGMs severed. Breaking strength (Newton) was normalized to the sectional area and MPa (Newton/mm^2) was used to quantitatively compare the tensile strength of CGMs from each group. Elongation at breakage was used to quantitatively compare the toughness of CGMs from each group. CGMs that severed within 10 mm of the gripped ends were not included in the analysis. Nine sets of valid data were obtained for each group.

2.16. Statistical analysis

Data were expressed as the means ± standard deviation (SD). Statistical data were analyzed by one-way ANOVA (Newman–Keuls multiple comparison test) assuming equal variance using GraphPad Prism software. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Cryopreservation effect on KCs viability

To evaluate the effect of various freezing solutions on cryopreserved KCs, we used PI staining to determine the percentage of necrotic cells and compared the results with those of a non-cryopreserved group. As shown in Fig. 1A–E, the viability assay revealed that 53.37 ± 2.76% of cells were viable in the DMSO group, 64.27 ± 0.80% in the 0.2 M T + D group, 68.43 ± 0.85% in the 0.4 M T + D group, 66.40 ± 0.66% in the 0.6 M T + D group and 97.07 ± 1.47% in the non-cryopreserved group. Cell viability between the non-cryopreserved group and any one of cryopreserved groups was significantly different ($p < 0.05$.). Cell viability between the DMSO-treated group and any one of the three groups treated with trehalose was significantly different ($p < 0.05$). Cell viability between 0.2 M T + D group and either of the other two trehalose-treated groups was also significantly different ($p < 0.05$). However, there was no significant difference between the 0.4 M T + D group and 0.6 M T + D group ($p > 0.05$) (Fig. 1K).

Results were confirmed by a LIVE/DEAD Viability/Cytotoxicity assay. Live cells were stained green and necrotic cells stained red (Fig. 1F–J). Cell viabilities were similar to those of the flow cytometric analysis.

3.2. Cryopreservation effect on KCs proliferation and cell cycle

To evaluate the effect of 1 month cryopreservation on KCs proliferation, daily DNA assays were performed for 9 days after thawing. As shown in Fig. 2A, all cultures reached a plateau phase at day 8. On day 4, cells of the non-cryopreserved group displayed a significantly higher cell number compared with that of any cryopreserved group ($p < 0.05$). The same trend was observed on day 5 ($p < 0.05$), while from day 6–9 there were no significant differences between non-cryopreserved and 0.4 M T + D or 0.6 M T + D groups ($p > 0.05$).

The cell number of the DMSO group at the plateau phase was the lowest of all groups, and there was a significant difference between the DMSO and non-cryopreserved or trehalose-treated groups ($p < 0.05$). At day 8, there was no significant difference between the non-cryopreserved group and the 0.4 M T + D or 0.6 M T + D groups ($p > 0.05$). Cell number of 0.4 M T + D group was slightly higher compared with that of the 0.6 M T + D group; however, there was no significant difference ($p > 0.05$).

To evaluate the percentage of proliferating cells, the cell cycle stage was assessed at days 1, 4, 7 and 9 after thawing. As shown in Fig. 2B, at day 1 the number of cells in S phase of the non-cryopreserved group showed a significant difference compared with each of the cryopreserved groups ($p < 0.05$). On day 4, the number of cells in S phase was obviously increased in all groups. There was a significant difference between the non-cryopreserved and DMSO groups and between the 0.4 M T + D and DMSO groups ($p < 0.05$). On days 7 and 9, the number of cells in S phase was decreased in all groups and there were no significant pairwise differences between groups ($p > 0.05$).

3.3. Cryopreservation effect on KCs marker expression

To characterize cryopreserved KCs *in vitro*, KCs markers were examined by immunofluorescent staining (Fig. 3A) and flow cytometric analysis (Fig. 3B and Fig. S1). pCK positive cell percentages in non-cryopreserved, DMSO, 0.2 M T + D, 0.4 M T + D and 0.6 M T + D

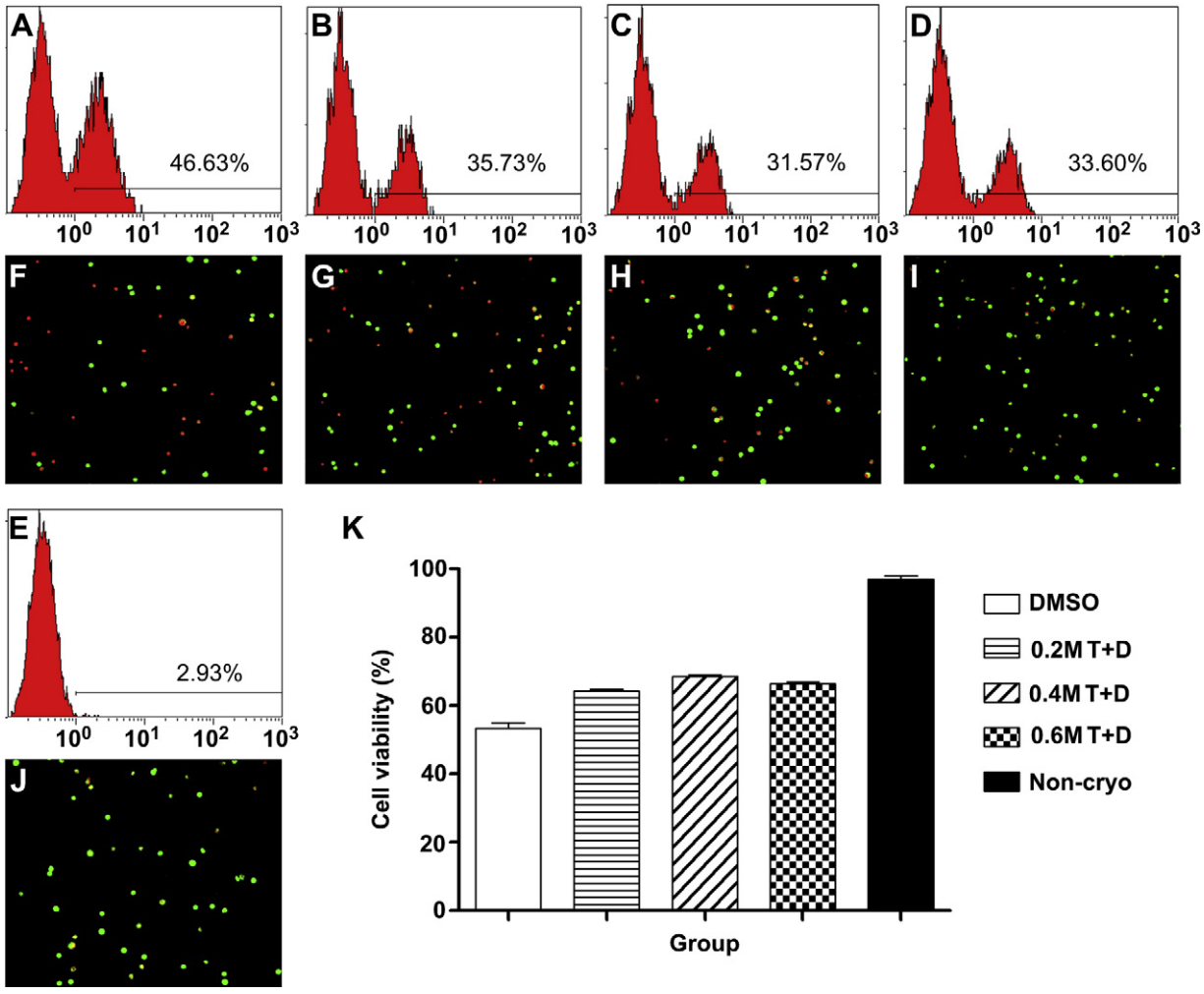

Fig. 1. Cell viability assays. (A–E) Flow cytometry results of PI staining to measure the non-viable KC percentages in groups A) DMSO, B) 0.2 M T + D, C) 0.4 M T + D, D) 0.6 M T + D, and E) non-cryopreserved cells. (F–J) Corresponding images below each flow cytometry graph show the results of cell viability staining for each group. (K) Overall viable cell percentages for each group. Error bars represent SDs, $n = 9$ for each group. Scale bar = 50 μm.

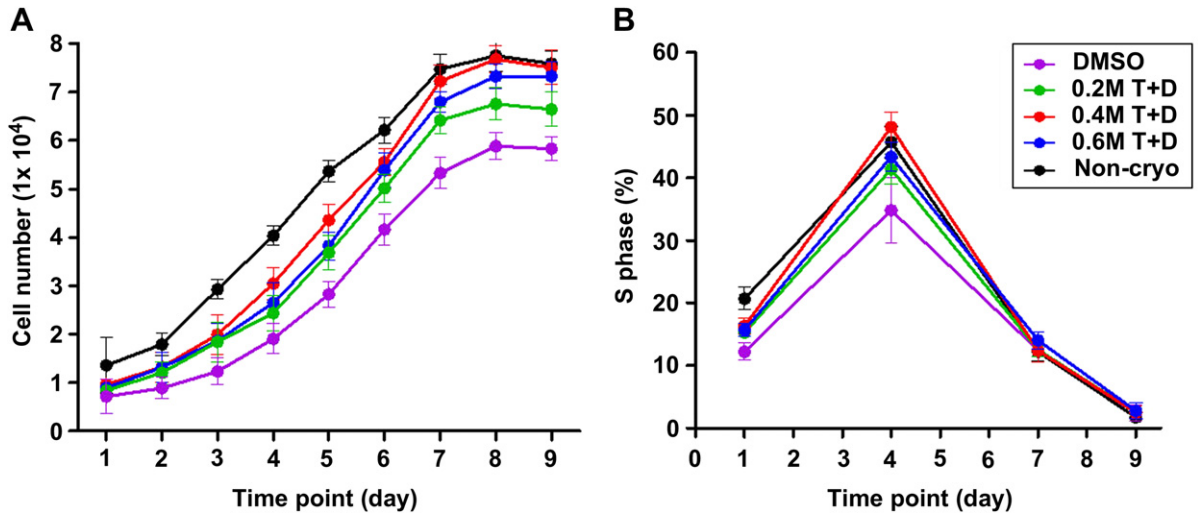

Fig. 2. Cell proliferation and cell cycle phase. (A) cell numbers at each time point from the various cryopreservation groups and non-cryopreserved control group. All groups entered a plateau phase at ~ day 8. (B) Cell percentages in S-phase after thawing at days 1, 4, 7, and 9 for the various groups as determined by DNA staining, $n = 9$ for each group.

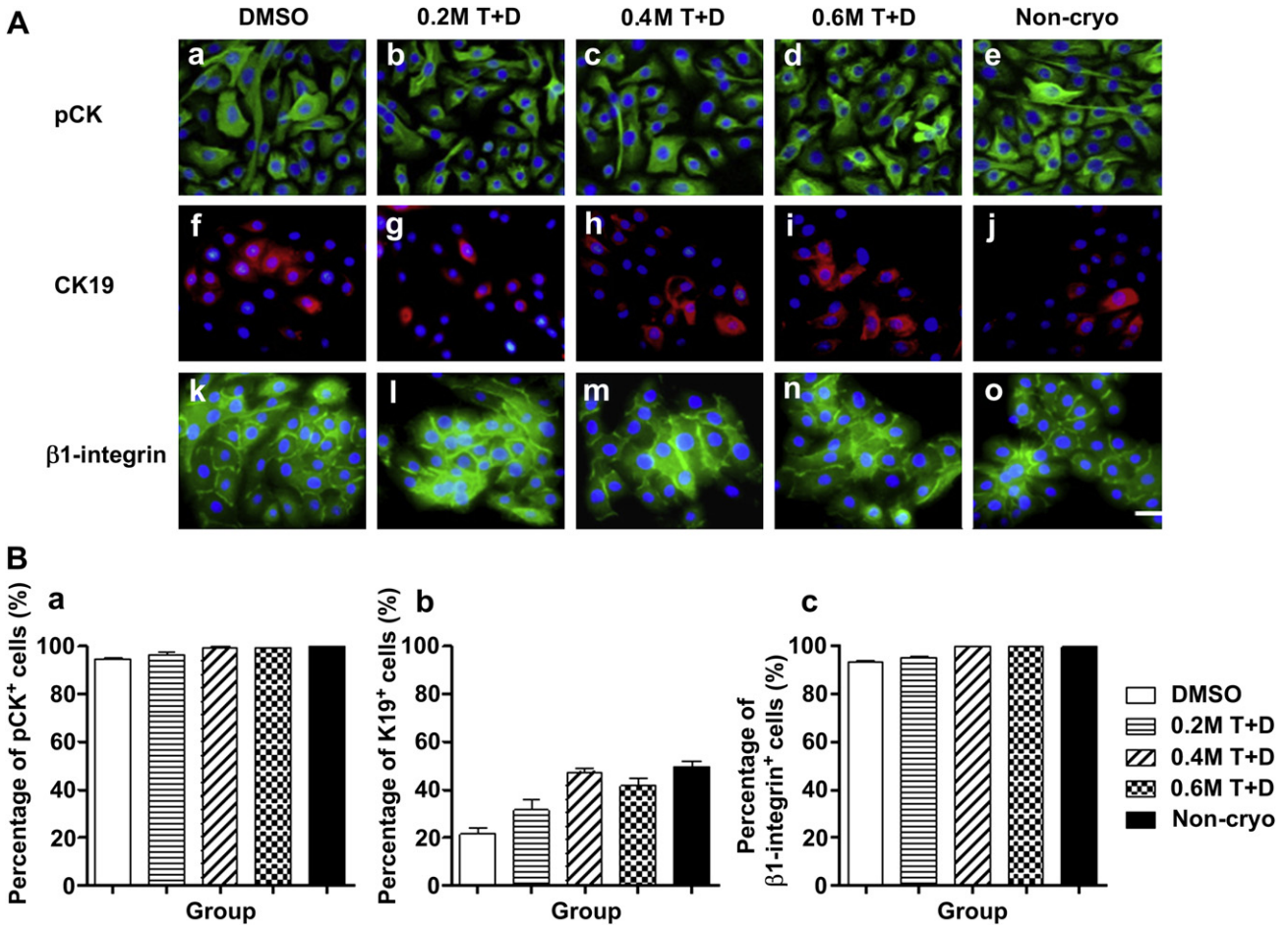

Fig. 3. KC marker expression. (A) Fluorescent images of cells expressing KC markers pCK, K19 and β1-integrin. (B) Representative fluorescence micrographs demonstrating staining for KC markers from each group, $n = 9$ for each group. Scale bar = 50 μm.

groups were 99.8 ± 0.1%, 94.5 ± 1.3%, 96.4 ± 1.1%, 99.6 ± 0.2% and 99.4 ± 0.3%, respectively. There was no significant difference between non-cryopreserved and 0.4 M T + D or 0.6 M T + D groups, and between 0.4 M T + D and 0.6 M T + D groups ($p > 0.05$). CK19 positive cells percentages in non-cryopreserved, DMSO, 0.2 M T + D, 0.4 M T + D and 0.6 M T + D groups were 49.5 ± 2.46%, 21.6 ± 2.54%, 31.5 ± 4.64%, 47.4 ± 1.65% and 41.8 ± 3.13%, respectively. Maximal expression was reached by the non-cryopreserved group, while there was no significant difference between non-cryopreserved and 0.4 M T + D groups ($p > 0.05$). β1-integrin positive cell percentages in non-cryopreserved, DMSO, 0.2 M T + D, 0.4 M T + D and 0.6 M T + D groups were 99.8 ± 0.1%, 93.2 ± 0.6%, 95.4 ± 0.9%, 99.8 ± 0.15% and 99.7 ± 0.2%, respectively. There was no significant difference between non-cryopreserved and 0.4 M T + D or 0.6 M T + D groups and between 0.4 M T + D and 0.6 M T + D groups ($p > 0.05$).

3.4. Trehalose improves cell viability of cryopreserved KC-CGMs

To evaluate the effect of various CPAs on cryopreserved KC-CGMs, we used the viability/cytotoxicity assay to determine viable cell percentages in DMSO and 0.4 M T + D groups as well as the non-cryopreserved control group (Fig. 4A–F). The cell viability assay showed that 95.9 ± 2.07% of cells were viable in the non-cryopreserved group, 31.2 ± 3.30% in the DMSO group and 57.4 ± 2.91% in the 0.4 M T + D group. Cell viabilities between DMSO and 0.4 M T + D groups were significantly different ($p < 0.05$). Comparing micrographs of the non-cryopreserved control with that of the cryopreserved KC-CGMs revealed that immediately after thawing (Day 0), cell morphology of the DMSO group was rounded and the cell number was obviously decreased. However, 0.4 M T + D group cells maintained their cobblestone morphology and cell number was also decreased. Three days after thawing, cell morphology of the DMSO group returned to a cobblestone morphology. Cell numbers of both cryopreserved groups increased; however, a 0.4 M T + D group was higher compared with that of the DMSO group.

SEM analysis (Fig. 4G–L) showed that there were a small number of pores on the DMSO group cell membrane immediately after thawing, while the 0.4 M T + D group membrane remained intact and was similar to that of the non-cryopreserved group. Three days after thawing, KCs on the CGMs in the 0.4 M T + D group were increased compared with that of the DMSO group.

3.5. Quantitative analysis of growth factor secretion from cryopreserved KC-CGMs

Growth factor secretion from fresh and cultured KC-CGMs for 1 and 3 days after thawing were determined. One day after thawing, EGF secreted from DMSO, 0.4 M T + D and non-cryopreserved groups was 0.07 ± 0.16 ng/mL, 0.17 ± 0.15 ng/mL and 1.23 ± 0.09 ng/mL, respectively. Three days after thawing, EGF secreted from DMSO and 0.4 M T + D groups was 0.17 ± 0.15 ng/mL and 0.72 ± 0.16 ng/mL, respectively. There was no significant

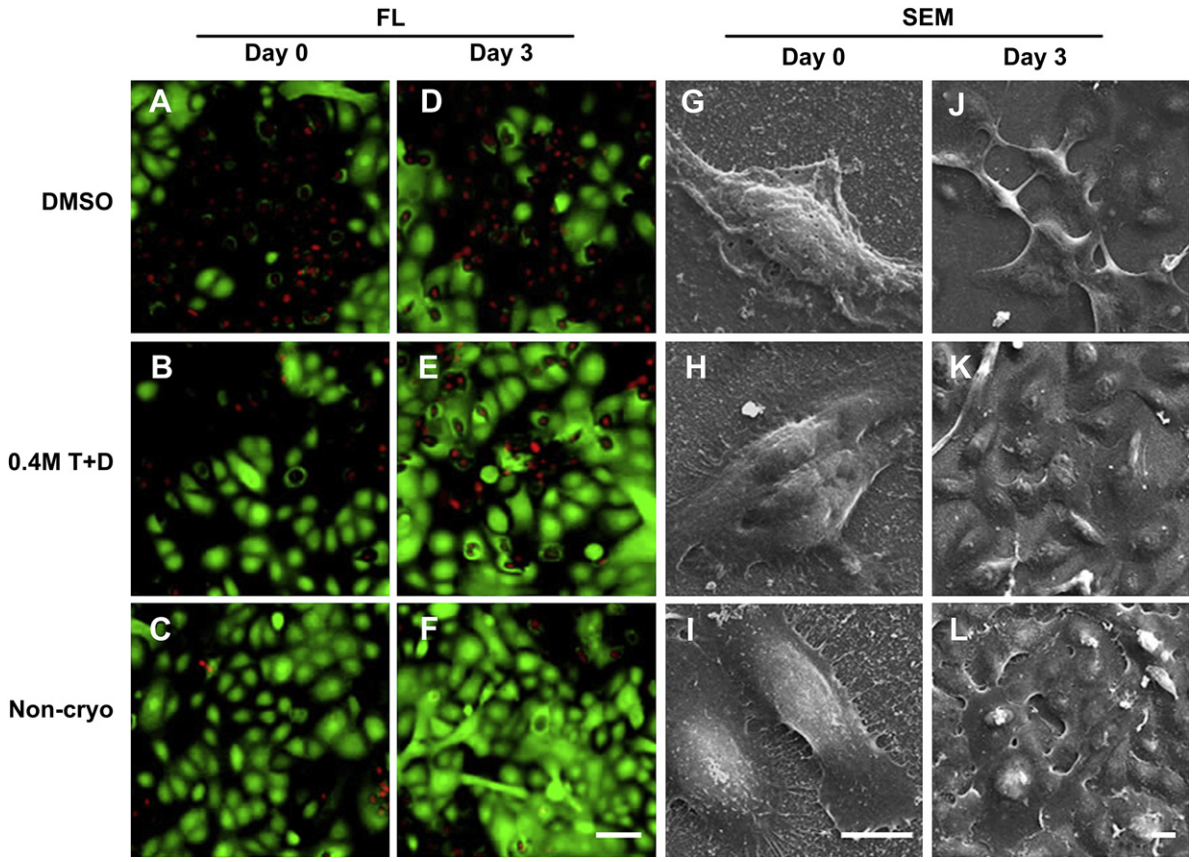

Fig. 4. KCs cultured on CGMs. Fluorescence (LIVE/DEAD stain) and SEM images of KCs seeded on CGM immediately after and 3 days following cryopreservation are shown with micrographs from a non-cryopreserved control, $n = 9$ for each group Scale bar in $F = 50$ μm for A–F, scale bar in I = 10 μm for G–I and scale bar in $L = 10$ μm for J-L.

difference in EGF secretion between DMSO and 0.4 M T + D groups one day after thawing ($p > 0.05$), while there was a significant difference between DMSO and 0.4 M T + D groups 3 days after thawing ($p < 0.05$) (Fig. 5A).

As shown in Fig. 5B, TGF-β1 secreted after 1 day of thawing from non-cryopreserved, DMSO and 0.4 M T + D groups was 124.14 ± 12.28 pg/mL, 80.01 ± 8.66 pg/mL and 118.13 ± 1.85 pg/mL, respectively. Three days after thawing, TGF-β1 secreted from DMSO and 0.4 M T + D groups was 87.46 ± 6.54 pg/mL and 128.09 ± 8.46 pg/mL, respectively. The non-cryopreserved group released a significantly higher TGF-β1 when compared with that of the DMSO group at either time point ($p < 0.05$). In addition, there was a significant difference between TGF-β1 secreted from DMSO and 0.4 M T + D groups at both time points ($p < 0.05$).

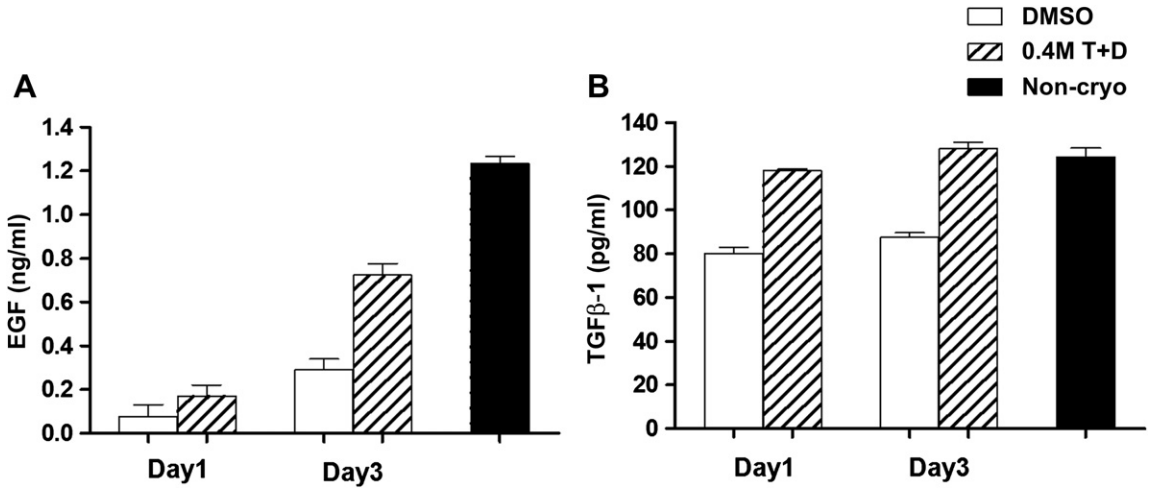

Fig. 5. Secretion of EGF (left) and TGF-β1 (right) from cryopreserved KCs, 1 and 3 days after thawing. Growth factor secretion from non-cryopreserved cells is also shown for comparison, $n = 9$ for each group.

3.6. Cryopreserved KC-CGMs promoted mouse wound healing in vivo

External appearances of representative experimental animals from fresh KC-CGMs, cryopreserved KC-CGMs with DMSO and 0.4 M T + D and CGMs alone groups were digitally recorded on days 0, 4,7,11 and 14 following surgery (Fig. 6A).

As shown in Fig. 6B, post operative wound area reduction was quantitatively measured. The CGMs alone (non-cryopreserved) reduced the wound size at the lowest rate and by day 14 the overall wound closure size was 63.3 ± 0.8%, which was significantly smaller compared with those of any other group ($p < 0.05$). The non-cryopreserved KC-CGMs group exhibited the greatest wound closure and was almost completely healed by day 14 (99.6 ± 0.56% wound area reduction). Wound repair decreased in both cryopreserved KC-CGMs groups. However, at day 11 the 0.4 M T + D group had significantly different wound healing (86.63 ± 0.80% wound area reduction) compared with that of DMSO alone (72.7 ± 0.46%) ($p < 0.05$). Similar to non-cryopreserved controls at day 14, the 0.4 M T + D group resulted in near complete wound resurfacing (94.4 ± 4.9%). DMSO KC-CGMs had wound healing of 86.33 ± 5.7%, which was significantly lower compared with those of trehalose or non-cryopreserved KC-CGMs groups ($p < 0.05$).

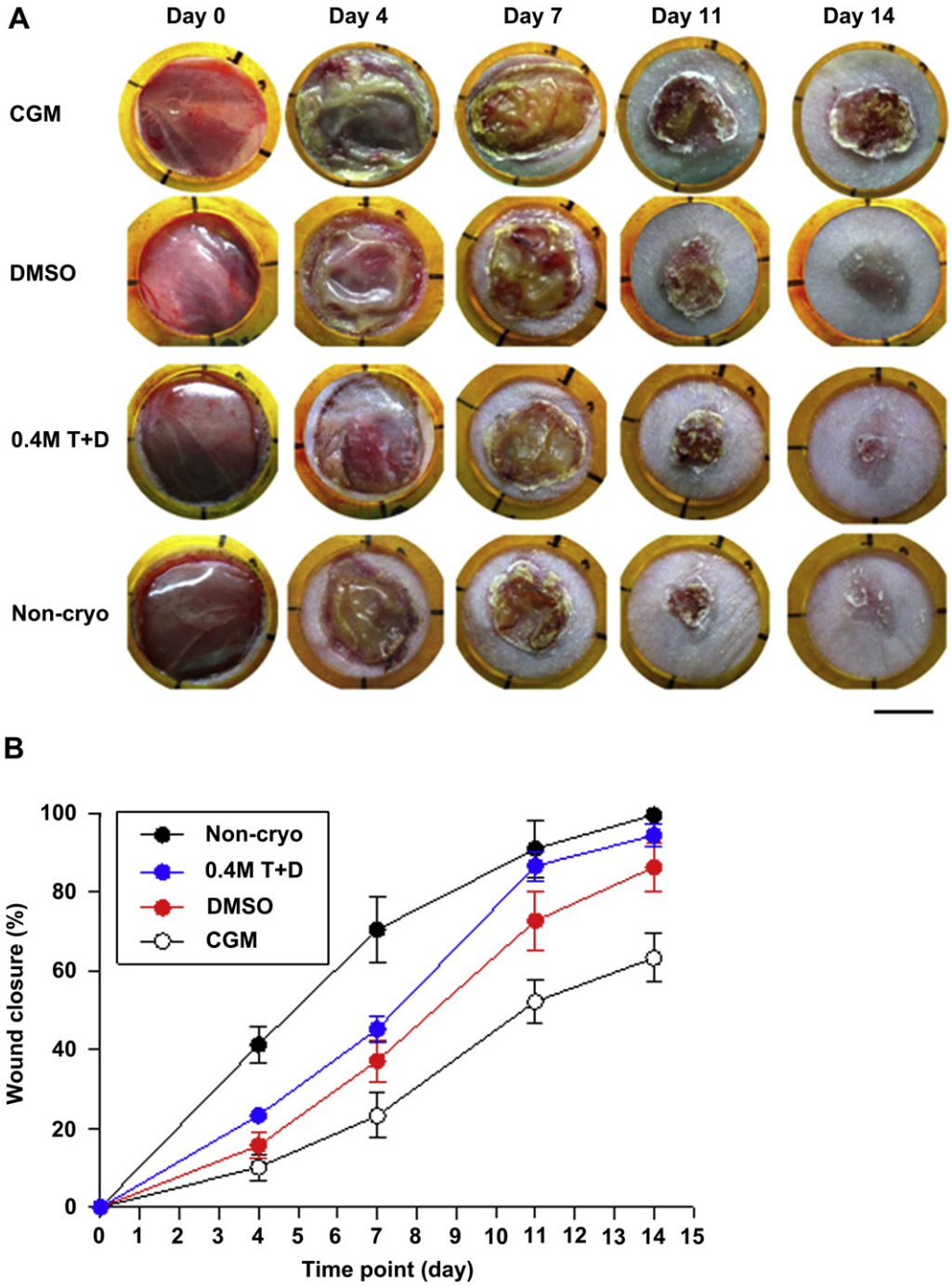

Fig. 6. *In vivo* wound healing. (A) Representative images of nude mice epidermal wounds are shown for each group and time point. Scale bar = 5 mm. (B) Mean wound closure percentage for each group over the time course Error bars represent SDs, $n = 9$ for each group.

To determine the KCs distributions *in situ*, regenerated skin tissue was harvested at day 14 and human KCs were detected with an anti-HLA-ABC antibody by immunohistochemistry using frozen sections. HLA-ABC immunostaining showed that human KCs existed in all KC-CGMs groups (Fig. 7A–K), while staining was not observed in the CGMs alone group and normal mouse skin (Fig. 7M–S). Epidermal thicknesses were 67.17 ± 8.15 μm in the non-cryopreserved group and 59.19 ± 5.53 μm in the 0.4 M T + D group with no significant difference between groups ($p > 0.05$). Epidermal thicknesses were 34.04 ± 5.17 μm in the DMSO group and 13.25 ± 3.71 μm in the CGMs group. There was a significant difference in the epidermal thicknesses between 0.4 M T + D and DMSO groups ($p < 0.05$). The epidermal thickness of nude mice skin was 25.02 ± 6.27 μm (Fig. S2).

3.7. Cryopreservation effect on biomechanical properties of CGMs

Examination with a biomechanical analyzer revealed that elongation at breakage of non-cryopreserved, DMSO and 0.4 M T + D groups were 289% ± 81%, 193% ± 29% and 212% ± 23%, respectively (Fig. 8A). Statistical analysis showed that elongation at breakage prior to cryopreservation was significantly higher compared with that of the two cryopreserved groups ($p < 0.05$), indicating that membranes are damaged to some degree by freezing and thawing phase transition. However, tensile strengths of the control, DMSO and 0.4 M T + D groups were 5.33 ± 1.49 MPa, 4.01 ± 1.06 MPa and 4.85 ± 1.44 MPa, respectively, and no significant difference was observed among three groups (Fig. 8B). SEM photographs of CGMs showed that a small number of fine cracks were observed on the membranes of DMSO group but not on the membranes of 0.4 M T + D group (Fig. 8C).

4. Discussions

To facilitate engineered epidermal substitutes for clinical treatment of wounds, such as burns, ulcers and cosmetic repairs, cryopreservation techniques that yield the optimal viability of epidermal membranes is highly advantageous. Although numerous advances have been made in human skin cryopreservation [10,11], there are few reports on the cryopreservation of cultured epithelial grafts [20]. In this study, we demonstrate the cryopreservation of engineered epidermis using various concentrations of trehalose combined with DMSO as cryoprotectant agents. This combination improved survival and proliferative capacities of cryopreserved KCs suspensions and engineered epidermis compared with that of DMSO alone. Moreover, transplanted engineered epidermal grafts that had been cryopreserved with trehalose/DMSO were indistinguishable from fresh control grafts and were superior to DMSO-cryopreserved grafts.

The most common cryoprotectant used by skin banks for human skin cryopreservation is DMSO. Generally, 10% DMSO is routinely used with 60–75% recovered viability of cultured human cells in

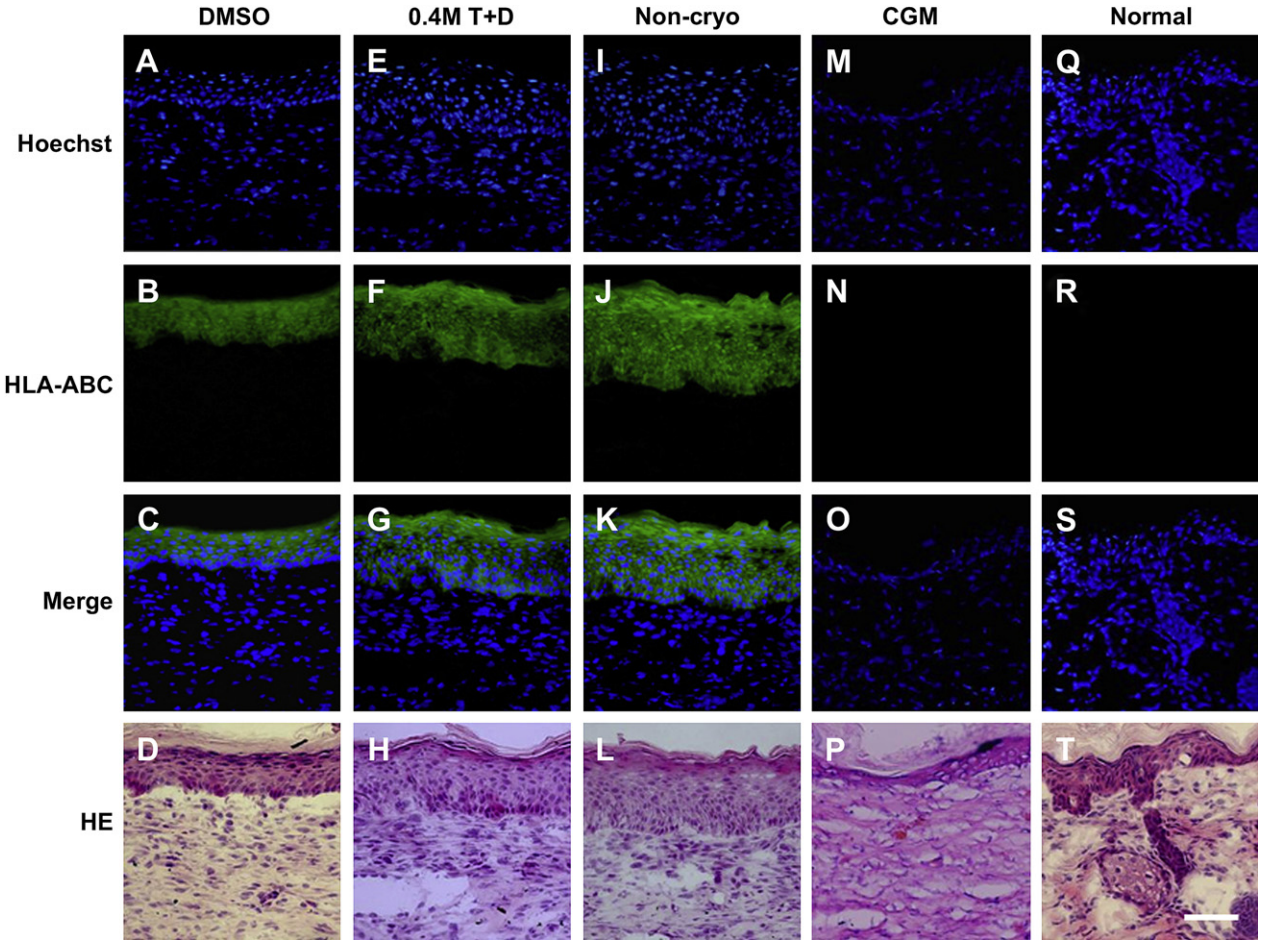

Fig. 7. *In vivo* graft incorporation. After day 14, grafts with the surrounding tissues were harvested and processed for immunohistochemistry. HLA-ABC staining indicates human keratinocytes and their progeny within mouse skin. Staining is absent in mice that received an acellular control membrane and intact control mouse skin. H&E staining is also presented (bottom row). Scale bar = 100 μm, $n = 9$ for each group.

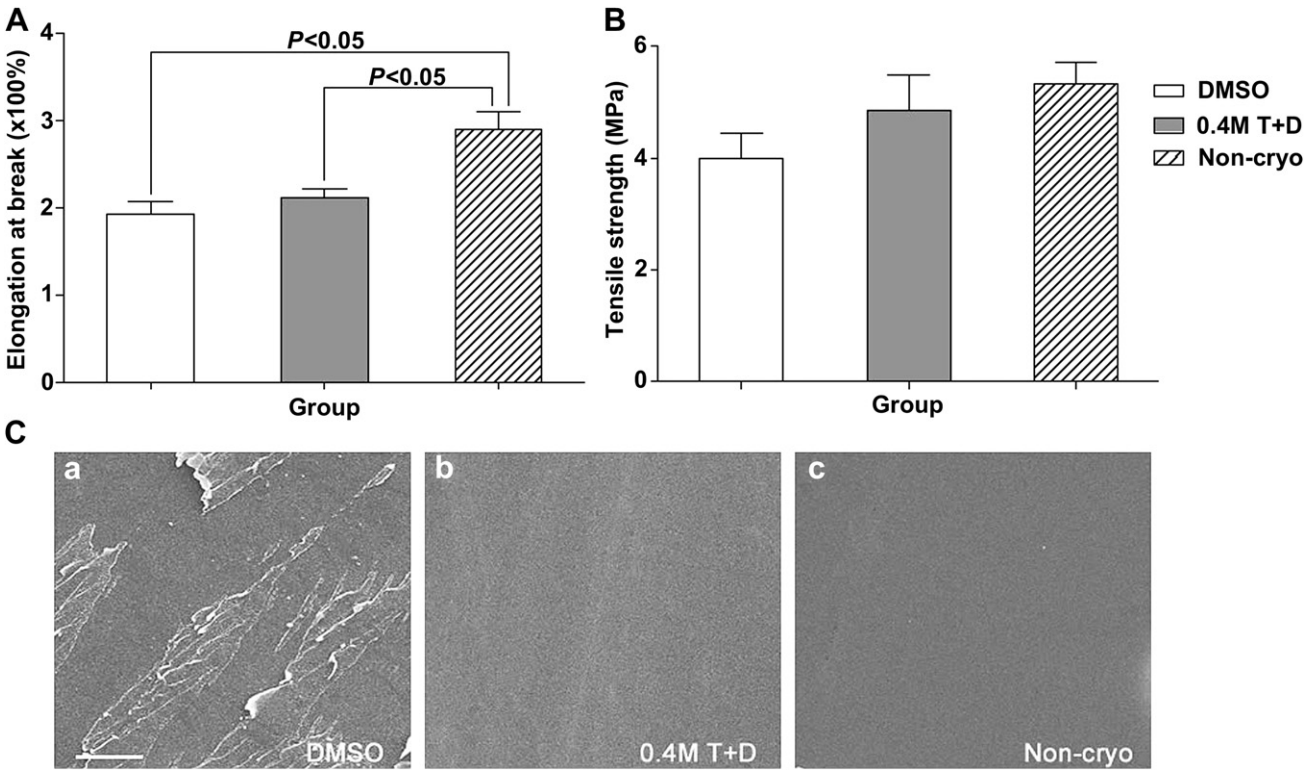

Fig. 8. Biomechanical properties and SEM images of CGMs. (A) Elongation at breakage of CGMs. (B) Tensile strengths of CGMs. (C) SEM images of CGMs. Scale bar = 10 μm, $n = 9$ for each group.

suspension [13]. In our study, a similar recovery rate (53.37%) was observed in KCs suspensions, while a low survival rate (31.2%) was observed in KC-CGMs epidermal grafts. These efficiencies were very close to that achieved by Pasch et al. [21]. Compared with KCs in suspension, KCs grown on the CGMs may not have been able to freely respond to osmotic changes due to scaffold adherence and cell–cell anchoring junctions. In addition, KCs may have a reduced surface area compared with that of suspended cells, which alters the exchange area for osmotic processes during cryopreservation [22]. Therefore, KCs cultured on CGMs result in a low survival rate after thawing.

Typically, addition of sugars to penetrating cryoprotectant (PC) in cryopreservation protocol is leading to improvement of results to some extent [23,24]. It is particularly apparent if results were compared with group that was cryopreserved with as little as 10% PC. Trehalose is a ubiquitous molecule present in lower and higher life forms but not mammals, and is synthesized as a stress-responsive factor by cells exposed to environmental stresses such as heat, cold, oxidation and desiccation. Unicellular organisms exposed to stress adapt by synthesizing large amounts of trehalose to assist in retaining cellular integrity [25]. Therefore, trehalose has been used as a non-permeating cryoprotectant for cryopreserving various cell types [24,26]. Trehalose is considered to prevent cellular protein degradation during freeze/thaw procedures [27]. 'Immobilization theory' states that trehalose acts as an insulator that confines biomolecules inside a matrix [28]. Trehalose is also considered a replacement for the water shell around proteins/membranes, which preserves their three-dimensional structures [29]. By dehydrating cells, trehalose also assists in avoiding excessive swelling and osmotic shock during removal of permeating cryoprotectants [16]. In addition, trehalose stabilizes membranes and proteins during freezing/dehydration [30,31]. In the current study, we demonstrated that trehalose significantly improves the recovery of KCs grown on CGMs. SEM analysis revealed that cell membranes remained intact in the 0.4 M T + D group (Fig. 4H), which was similar to non-cryopreserved group (Fig. 4I), while a small number of pores were observed on cell membranes in the DMSO group (Fig. 4G), suggesting that trehalose may protect KCs by stabilizing cell membranes. Moreover, trehalose has been shown to have excellent glass-forming properties [32]. Employment of trehalose+10%DMSO might support small proportion of amorphous inclusion. It is known that vitrification is the best option for cryopreservation as it avoids ice crystal formation during vitrification/thawing procedures in cell-biomaterial constructs entirely [33,34]. However, when commonly used vitrification solution VS55 was utilized to vitrify KCs and KC-CGMs, only 10% of cells were survived after thawing (unpublished data). The relatively high solution concentration of VS55 is likely toxic to KCs. We therefore tested different concentration of trehalose for freezing KCs, and trehalose at 0.4 M showed best effects. It has been reported that the optimal concentration of trehalose could be different from cell to cell. In cryopreservation of human hematopoietic cells from umbilical cord blood, 0.06 M of trehalose was optimal [35] , while in human ES cells and fetal skin, 0.2 M and 0.5 M of trehalose were better [16,17]. Clearly, the concentration of trehalose for cryopreservation is cell type dependent.

Wound repair processes are initiated immediately after injury by various growth factor and cytokine secretions, cellular proliferation, neovascularization, extracellular matrix deposition and reorganization that leads to healing and restoration of tissue integrity [36]. The improvement of *in vivo* wound healing in the trehalose group was anticipated due to results from *in vitro* studies. Trehalose not only improves cell survival but also preserves EGF and TGF-β1 secretion from KCs. HLA-ABC staining confirmed the contribution of human KCs in the wound healing process. In our previous clinical trial, we found that allogeneic KCs can survive on

the wound bed for at least 10 days, which promotes wound healing at split-thickness graft donor sites and reduces long term hypertrophic scarring [9].

The mechanical properties of CGMs before and after cryopreservation were also investigated. Elongation at breakage as a quantitative parameter for the toughness of CGMs decreased significantly in DMSO and 0.4 M T + D groups. CGMs tensile strength is the maximum tensile stress that can be applied before failure and was also decreased in DMSO and 0.4 M T + D groups. The decrease of mechanical properties might due to the ice punches and cracks on the membrane during freezing and thawing processes. However, SEM analysis only showed a small number of fine cracks on several membrane pieces in the stepped freezing group, while no punch was observed on the membranes (Fig. 8C). In addition, cryopreservation procedure does not affect the adhesion property of CGMs (data not shown). Although the mechanical properties of CGMs decreased after cryopreservation, the membranes remained strong enough to be handled. KC-CGMs that are placed into liquid nitrogen become brittle and can be easily broken in the freezing bag by external forces during thawing (data not shown). To protect from external forces, we used cryovials to contain individual KC-CGMs pieces. Thus, membrane integrity was protected during cryopreservation and thawing procedures.

5. Conclusions

In summary, we established a cryopreservation protocol for tissue-engineered epidermal membranes using trehalose in combination with DMSO as the cryoprotectant. KCs survival is significantly improved using this protocol that may accelerate the application of tissue-engineered products in the clinic.

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Appendix. supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2011.07.008.

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